Composition and effect of blending of noncoagulating, poorly coagulating, and well-coagulating bovine milk from individual Danish Holstein cows

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ABSTRACT

The aim of the present investigation was to study the underlying causes of noncoagulating (NC) milk. Based on an initial screening in a herd of 53 Danish Holstein-Friesians, 20 individual Holstein-Friesian cows were selected for good and poor chymosin-induced coagulation properties; that is, the 10 cows producing milk with the poorest and best coagulating properties, respectively. These 20 selected cows were followed and resampled on several occasions to evaluate possible changes in coagulation properties. In the follow-up study, we found that among the 10 cows with the poorest coagulating properties, 4 cows consistently produced poorly coagulating (PC) or NC milk, corresponding to a frequency of 7%. Noncoagulating milk was defined as milk that failed to form a coagulum, defined as increase in the storage modulus (G') in oscillatory rheometry, within 45 min after addition of chymosin. Poorly coagulating milk was characterized by forming a weak coagulum of low G' . Milk proteomic profiling and contents of different casein variants, ionic contents of Ca, P and Mg, κ -casein (CN) genotypes, casein micelle size, and coagulation properties of the 4 NC or PC samples were compared with milk samples of 4 cows producing milk with good coagulation properties. The studies included determination of production of caseinomacropeptide to ascertain whether noncoagulation could be ascribed to the first or second phase of chymosin-induced coagulation. Caseinomacropeptide was formed in all 8 milk samples after addition of chymosin, indicating that the first step (cleavage of κ -CN) was not the cause of inability to coagulate. Furthermore, the effect of mixing noncoagulating and well-coagulating milk was studied. By gradually blending NC with well-coagulating milk, the coagulation properties of the well-coagulating sam-

ples were compromised in a manner similar to titration. Milk samples from cows that consistently produced NC milk were further studied at the udder quarter level. The coagulation properties of the quarter milk samples were not significantly different from those of the composite milk sample, showing that poor coagulation traits and noncoagulation traits of the composite milk were not caused by the milk quality of a single quarter. The milk samples exhibiting PC or NC properties were all of the κ -CN variant AA genotype, and contained casein micelles with a larger mean diameter and a lower fraction of κ -CN relative to total CN than milk with good coagulation properties. Interestingly, the relative proportions of different phosphorylation forms of α -CN differed between well-coagulating milk and PC or NC milk samples. The PC and NC milk samples contained a lower proportion of the 2 less-phosphorylated variants of α -CN (α_{S1} -CN-8P and α_{S2} -CN-11P) compared with samples of milk that coagulated well.

Key words: noncoagulating milk, coagulation property, casein phosphorylation, caseinomacropeptide

INTRODUCTION

The 6 major proteins found in bovine milk comprise the 4 caseins, α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN, together with the 2 whey proteins, α -LA and β -LG. To a large extent, the composition of these proteins in milk determines the nutritional value and technological properties of milk (Dalglish, 1993). Cheese yield increases with casein concentration, and technological properties such as rennet coagulation time and curd firmness depend mainly on casein composition and distribution (Wedholm et al., 2006; Hallén et al., 2007). Protein composition is largely determined by genetic factors, but it also varies with season, lactation day, parity, feeding, and health status of the cow (Schütz et al., 1990; Bobe et al., 1998). The caseins in milk assemble into micelles, large colloidal particles with diameters ranging from <50 nm to >500 nm and an

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average diameter of approximately 200 nm as measured by dynamic light scattering (Horne, 2003). The size of the casein micelle, the contents, and the distribution of ions and caseins are important for the coagulation properties of milk; for example, clotting time and curd firmness (Ekstrand et al., 1980; Dalglish, 1993; Glantz et al., 2010). Rennet coagulation of milk is very important, because it is the foundation of the cheese-making process. During renneting, κ -CN is cleaved by rennet enzymes such as the endopeptidase chymosin (EC 3.4.23.4). The enzymatic coagulation of milk is divided into 2 phases that are separate in nature although not entirely in time. The primary phase is the enzymatic cleavage of κ -CN between the residues Phe105 and Met106. Thereby, caseinomacropeptide (CMP; κ -CN peptide 106–169), which is the highly hydrophilic part of the C-terminal of κ -CN, is released into the whey. This process destabilizes the casein micelles by reducing the steric protection provided by the negatively charged CMP (Chaplin and Green, 1980; Sandra et al., 2007). The secondary phase of coagulation is non-enzymatic. When approximately 85% of the κ -CN is hydrolyzed, the destabilized casein micelles normally start a calcium-dependent spontaneous aggregation, resulting in a gel-like network—the curd or coagulum (Green and Morant, 1981; Fox and McSweeney, 1998), which contains the N-terminal part of κ -CN (para- κ -CN, κ -CN peptide 1–105).

The caseins are encoded by highly polymorphic genes that are tightly linked and clustered on bovine chromosome 6, and more than 35 different milk protein variants have been identified (Threadgill and Womack, 1990; Farrell et al., 2004; Caroli et al., 2009). The different genetic combinations of the 6 major milk proteins (i.e., the haplotypes) have a major effect on milk composition and coagulation properties (see reviews by Martin et al., 2002; Caroli et al., 2009). Because the coagulation of milk is crucial for cheese manufacturing, with influence on both cheese quality and yield, it is important to determine the occurrence and underlying reasons for poorly coagulating (PC) and noncoagulating (NC) milks from individual cows of different dairy breeds and to evaluate the effects of blending milks with different coagulation properties. The phenomenon of NC milk has been increasingly studied during recent years (Okigbo et al., 1985a; Ikonen et al., 1999; Wedholm et al., 2006; Joudou et al., 2007), and both genetic and environmental factors are associated with NC milk. In general, the genetic factors play an important role in determining milk coagulation ability, and the positive influence of the B alleles of both κ -CN and β -CN has been well established (Hallén et al., 2008). In contrast, the κ -CN A and E alleles are associated with poor coagulation properties (Ikonen et al., 1999; Hallén et al.,

2007). Cows with the κ -CN E allele have been associated with NC milk on several occasions (Ikonen et al., 1999; Caroli et al., 2009; Wedholm et al., 2006), and recently, 2 candidate genes associated with NC milk were identified in Finnish Ayrshire cows (Tyriseva et al., 2008).

However, the genetic contribution is only partly responsible for the occurrence of NC milk, as NC milk has also been found to be associated with cows in mid or late lactation and with cows having mastitis (Okigbo et al., 1985a,c; Ikonen et al., 2004).

The aim of the present study was to investigate the frequency of PC or NC milk in a herd of Danish Holstein-Friesian cows, to compare the composition of such milk with well-coagulating (WC) milk, and to identify whether the failure in coagulation properties originated in the first or second phase of the coagulation process. Furthermore, the aim was to evaluate the effect of blending PC or NC milk with WC milk, and to study whether the poor coagulation properties of the composite milk from an individual NC cow were the result of very poor coagulation properties in the milk from one gland only or in milk from all 4 glands of the udder.

MATERIALS AND METHODS

Animals

The cows were housed in a loose housing system with a free-cow traffic system and milked by an automatic milking system (Voluntary Milking System, DeLaval, Tumba, Sweden) as described previously (Frederiksen et al., 2011). The cows were fed ad libitum with a TMR and supplemented with concentrate according to standard practice. Lactation stage (weeks and days), parity (1–4), daily milk yields (kg), milk yield (kg) in the individual milking, and time since last milking (h) were recorded for each milk sample collected.

Milk Samples

Eight individual cows of the Danish Holstein-Friesian breed from the resident herd at the Danish Cattle Research Center (Tjele, Denmark) were selected from screening among 53 cows (Frederiksen et al., 2011). Based on the milk coagulation properties of milk samples taken individually from the 53 cows, 10 cows producing WC milk and 10 cows producing PC milk were selected, followed, and sampled another 6 to 10 times over 7 mo (Figure 1). Four of the 10 cows producing poorly coagulating milk consistently produced milk with poor coagulation properties during the follow-up period and were selected for further analysis. Two of

Figure 1. Cow selection outline and definition of coagulation groups. Milk samples from 53 individual Danish Holstein-Friesian cows were collected and evaluated for coagulation properties. The rennet coagulation time (RCT), curd firming rate (CFR), and gel firmness of the coagulum were measured by using dynamic rheological analysis. Based on this, 10 cows producing well-coagulating milk and 10 cows producing poorly coagulating milk were selected. In a 7-mo follow-up period, the cows were sampled another 6 to 10 times. Four of the 10 cows producing poorly coagulating milk were found to consistently produce milk with poor coagulation properties (2 of these with poor coagulation and 2 with noncoagulation) during the follow-up period and were selected for further analysis. Four cows producing well-coagulating milk were selected for comparison.

the cows in this group consistently produced NC milk. In parallel, 4 cows consistently producing WC milk were selected as donors of WC milk for comparison.

Individual milk samples from the 8 selected cows were collected from whole morning milks on a single occasion. The milk samples were immediately refrigerated at 5°C for 1 to 4 h, without preservation, before evaluation of coagulation properties. Each milk sample was analyzed for concentrations of milk fat, protein, casein, lactose, citric acid, and urea by using Milkoscan FT 6000 (Foss Electric, Hillerød, Denmark) and for SCC using Fossomatic 5000 (Foss Electric) at Eurofins Laboratory (Holstebro, Denmark). Udder milk samples were collected in separate containers from each quarter, but otherwise handled and measured as stated above.

Determination of Ionic Contents of Phosphorus, Calcium, and Magnesium

The content of P in milk samples was determined by the vanadomolybdate colorimetric procedure (Stuffins, 1967), and the contents of Ca and Mg by atomic

absorption spectrophotometry (Atomic Absorption System SP9, Pye Unicam Ltd., Cambridge, UK). The separation into soluble and colloidal phases was made by high-speed centrifugation of 200 mL of fresh skim milk at $100,000 \times g$ for 1 h using a T4-TI-70 rotor in a Beckman-Coulter Optima L-80XP Ultracentrifuge (Beckman Coulter Inc., Brea, CA). Because temperature influences equilibrium of ions between soluble and colloidal phases and is important for chymosin-induced coagulation, the separation of soluble and colloidal phases by high-speed centrifugation was performed at 33°C, the temperature used for the coagulation studies. The supernatant fluid was carefully removed and analyzed for Ca, Mg, and P contents representing the soluble fraction hereof. The total contents of Ca, Mg, and P were measured in skim milk samples. The colloidal content of ions was calculated as the difference between total and soluble contents. All samples were measured in duplicate, and the ionic contents are presented as means of measurements.

Casein Micelle Size

Fresh milk obtained from the 8 selected cows was centrifuged twice at $200 \times g$ for 20 min at 4°C. Skim milk samples were diluted 1:100 in milk ultrafiltrate (MUF), equilibrated to 33°C, and filtered through a 0.22- μ m syringe filter before analyses. The MUF was prepared at the dairy plant (Nr. Vium, Denmark) by ultrafiltration of tank milk at 8°C according to standard procedure at the dairy. The average diameter and size distribution of casein micelles in the filtered skim milk samples diluted in MUF were determined by dynamic light scattering using a Malvern Nano S ZEN1600 Zetasizer from Malvern Instruments (Worcestershire, UK). The particle size measurements were conducted at a fixed 173° angle and a wavelength of 633 nm. The milk to solvent ratio was 10 μ L of skim milk per 1 mL of MUF solvent, as required by the expert advice system in the Zetasizer Nano Software for aiding interpretation of data and results (Dispersion Technology Software 5.10, Malvern Instruments Ltd.). The particle size measurements were performed at a constant temperature of 33°C, which was chosen to match the conditions for the rheological measurements. One milliliter of sample was loaded in disposable cuvettes (DTS 0012), and equilibrated for another 2 min at 33°C in a temperature-controlled chamber of the particle size analyzer, and the DLS measurement was promptly started. Data collection and analysis were performed using the dispersion technology software. Cumulative analysis of the intensity autocorrelation function was used to derive an average value and distribution of diffusion coefficients, which was converted to hydrodynamic diameters us-

ing the Stokes-Einstein equation for spherical particles. Five replicate measurements were performed for each experimental sample, and each measurement consisted of 11 subsequent individual runs of 10 s duration. For each measurement, the hydrodynamic diameters (nm), the relative particle size distribution, and the polydispersity index were determined.

Dynamic Rheological Analysis

Measurements of the coagulation properties [rennet coagulation time (RCT), curd firming rate, and coagulum firmness] were performed by continuous measurement during renneting using a ReoRox4 rheometer (MediRox AB, Nyköping, Sweden) as described by Frederiksen et al. (2011). Ten milliliters of milk was skimmed by centrifugation at $200 \times g$ for 20 min at 4°C and adjusted to pH 6.5 with 10% (vol/vol) lactic acid. The milk sample was preincubated for 30 min in a water bath at 33°C before addition of 0.038 international milk clotting units/mL of milk of ChyMax Ultra (Chr. Hansen Laboratories A/S, Hørsholm, Denmark). Rheological measurements were performed at 33°C for 40 min.

Blends of 2 selected skim milk samples from individual cows, one with NC properties and one with WC properties, were prepared by gradually increasing the content of NC milk from 0 to 5, 25, 50, 75, 95, and 100% (vol/vol). In addition, skim milk from the 4 cows in each coagulation property group was mixed in equal amounts to make 2 milk pools, one of poor and one of good coagulation quality. From these 2 milk pools, 5 samples with a PC milk pool content of 5, 25, 50, 75, and 95% were prepared. The pH values of the blends and pools were measured and adjusted to pH 6.5 before rheological analysis as described above.

The effect of Ca on the coagulation properties of the NC milk samples was evaluated by addition of CaCl_2 to a final concentration of 0.45 mM (corresponding to 0.005% wt/vol) to aliquots of these milk samples before the dynamic rheological analysis in a parallel experiment.

The RCT (min), the maximum coagulum firmness by storage modulus G' ($G'_{\max}$; Pa) and curd firming rate $[\Delta G'/\Delta t]_{\text{lin}}$ (Pa/min) were selected to describe the coagulation properties of the individual milk samples, essentially as described earlier (Frederiksen et al., 2011). The RCT was defined as the time where the phase angle $\theta = 45^\circ$. The curd firming rate rate $[\Delta G'/\Delta t]_{\text{lin}}$ was calculated from consecutive points of the linear part of the gelation profile, and the $G'_{\max}$ value equals the maximum value of the storage modulus G' obtained within the 40 min of measurement. Although the ReoRox4 instrument was developed with blood coagula-

tion in mind and reports moduli in Pascals, we found the reported moduli when measuring milk coagulation to be considerably higher than previously obtained using traditional controlled stress or strain rheometers. The reported values in Pascals in this paper equal the earlier reported values in ReoRox units (Frederiksen et al., 2011). All dynamic rheological analyses were carried out in duplicate.

Sampling During Renneting for Determination of CMP

During the dynamic rheological measurements, 1-mL aliquots of milk were withdrawn at different time points for isolation of CMP (0, 25 s, and 2, 5, and 10 min). The enzymatic reaction was stopped in all the withdrawn aliquots by addition of 10 μL of pepstatin stock solution [1 mg of pepstatin A (Sigma, St. Louis, MO) per milliliter of 10% (vol/vol) acetic acid in methanol] and placed on ice. The CMP was isolated by acid precipitation, and the renneted milk aliquots (1 mL) were carefully mixed with 100 μL of acetic acid (CH_3COOH ; 10%). After 2 min of incubation on ice, 100 μL of sodium acetate (CH_3COONa ; 1 N) was added, to pH 4.6, and the solution was mixed and centrifuged for 10 min at $1,000 \times g$ at 5°C. The supernatants containing the CMP were isolated, and the CMP concentration was determined by reverse phase (RP)-HPLC.

Determination of CMP by RP-HPLC

The CMP contents were determined by RP-HPLC essentially as described elsewhere (Minikiewicz et al., 1996). A linear gradient of solvent A (acetonitrile:water:trifluoroacetic acid 100:900:1, vol/vol/vol) in solvent B (acetonitrile:water:trifluoroacetic acid 900:100:0.8, vol/vol/vol) was applied. The gradient was set to start with 15% of solvent B and subsequently increase from 15 to 28% in 15 min, 28 to 32% in 22 min, 32 to 70% in 3 min, and then remain at 70% for 5 min before returning to the initial conditions. The separations were carried out at 30°C, with a flow rate of 1 mL/min, peak detection at 220 nm, with an injection volume of 100 μL , and using a Phenomenex reversed phase column (Jupiter 4 μM Proteo 90 Å New Column 250 $\times$ 4.6 mm, Torrance, CA). A commercial product of CMP (Lacprodan CGMP-20, Arla Foods amba, Global Ingredients, Nr. Vium, Denmark) with a declared CMP content >95% (wt/wt, DM basis) and a glyco-/aglyco-CMP ratio of 1:1 was used for making standard samples of 2.7 mg/mL. These samples were freshly prepared by dissolving the appropriate amount of powder in Milli-Q ultrapure water (Millipore, Billerica, MA); they were included before every supernatant sample run to serve as an

external reference. All samples and solutions were filtered through a 0.45- μ m filter (Frisenette Aps, Knebel, Denmark) before HPLC analysis.

The HPLC analysis was carried out on an HP 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (detection at 220 and 280 nm). Relative quantification of peaks was performed by automated integration of peak areas, and when required, by manual integration, using the Agilent ChemStation software Rev.B.03.01. The spectra monitored at 280 nm were used for identification of peaks not containing CMP, because CMP does not contain tryptophan or tyrosine residues and therefore has no UV absorbency at 280 nm, and these peaks were omitted from the integration. By referring to the standard, CMP was found to elute between 8 and 23 min, and the sum of integrated peaks at 220 nm in this time interval from chromatograms of the standard (Lacprodan CGMP-20) represented the total content of CMP in the standard. The CMP content in the supernatant samples is presented as ratio of the summed, integrated peak areas at 220 nm in each sample in that time interval relative to that of the standard:

$$(\text{CMP}_{\text{rel}} = \text{Area}_{\text{sample220nm}} / \text{Area}_{\text{standard220nm}}).$$

Determination of κ -, α -, and β -CN Variants by Liquid Chromatography/Electrospray Ionization-Mass Spectrometry

The reverse phase chromatography for separation and quantification of milk proteins applied in this study was developed based on previous studies (Bobe et al., 1998; Bonfatti et al., 2008; Bonizzi et al., 2009). Frozen skim milk samples were thawed at room temperature and mixed 1:1 (vol/vol) with a solution containing 6 M guanidine-HCl and 100 mM Bis-Tris, pH 7. Freshly prepared dithiothreitol was added to a final concentration of 10 mM. After incubation for 1 h at room temperature, the samples were centrifuged at $6,000 \times g$ using a tabletop centrifuge (Eppendorf 5417R Refrigerating Microcentrifuge, Enfield, NY) thermostatically controlled at 4°C. Prior to analysis, the samples were filtered through a 0.45- μ m polytetrafluoroethylene filter (Mini-Uniprep, Whatman, Florham Park, NJ). An HP 1100 system (Agilent Technologies) with a Jupiter C4 column (250 mm $\times$ 2 mm, 5- μ m particle size, 300 Å pore size, Phenomenex) and a diode array detector coupled to a mass selective detector was used for identification and relative quantification of milk proteins. The HPLC equipment consisted of a G1375 degasser (Agilent Technologies) connected to a G1312A binary pump. A G1315A diode array detector was used for UV detection at 214 nm. A relative quantification of

peaks was performed by automated integration of peak areas. Column temperature was kept constant at 35°C using a G1316A thermostatically controlled column compartment. Protein separation was conducted by using a binary solvent system with a linear gradient of solvent A (water:trifluoroacetic acid 999:1, vol/vol) in solvent B (acetonitrile:trifluoroacetic acid 999:1, vol/vol). The column was equilibrated at 31% solvent B, and immediately after sample injection (2 μ L), a linear gradient program was initiated from 31% B to 45% B with an increase of 0.3% B/min. At 45% B, the column was cleaned by injection of 2 μ L of water, and impurities were removed by isocratic elution at 80% B for 10 min. The column was recalibrated at 31% B before the next sample injection. The samples were analysed in triplicate. The HPLC systems and the mass selective detector were controlled using ChemStation software (rev. B.04.01 SP1 [650], Agilent Technologies). The settings of the electrospray ionization (ESI) source and the mass selective detector were as follows (ChemStation nomenclature in parentheses): ion mode (polarity): positive; capillary exit voltage (fragmentor): 200 V; capillary voltage: 4,500 V; nitrogen drying gas flow: 10 L/min; nitrogen drying gas temperature: 300°C. Masses (Da) of the milk proteins were obtained using the deconvolution algorithm of the ChemStation software.

Statistical Analysis

Statistical analysis was performed to calculate mean values and standard deviations for the various milk components, production parameters, and coagulation properties using SAS software program (version 9.2; SAS Institute Inc., Cary, NC). The *F*-test was performed to evaluate differences between the 2 groups of cows with different milk coagulation quality using a significance level of 95% (i.e., $P < 0.05$).

RESULTS

Rheological Properties of Milk Samples

Milk from the 8 cows representing 3 coagulation class groups, NC, PC, and WC, were selected based on the milk coagulation properties of milk samples taken individually from a herd of 53 cows (Frederiksen et al., 2011) as outlined in Figure 1. The milk samples referred to as WC milk were characterized by having an onset of coagulation within 15 min (RCT <15), curd firming rates >25 Pa/min, and maximum G' values >350 Pa (Table 1). Milk samples designated as PC did not start coagulation within 15 min, formed a curd at a rate <21 Pa/min, and obtained gel-firmness values of <350 Pa within the 40 min of measurement. The WC and PC groups were statistically differentiable

Table 1. Milk composition and production traits (SD in parentheses) as function of milk coagulation classes

Trait	Coagulation property class ¹		Significance based on <i>F</i> -test
	Good (n = 4)	Poor (n = 4)	
Total Ca (mg/kg)	1,349 (193)	1,210 (119)	NS
Total P (mg/kg)	944 (98)	906 (99)	NS
Total Mg (mg/kg)	117 (14)	119 (17)	NS
Micelle-bound Ca (mg/kg)	883 (156)	782 (92)	NS
Micelle-bound P (mg/kg)	505 (83)	459 (79)	NS
Micelle-bound Mg (mg/kg)	31 (7)	31 (8)	NS
Soluble Ca (mg/kg)	466 (68)	429 (56)	NS
Soluble P (mg/kg)	438 (37)	448 (54)	NS
Soluble Mg (mg/kg)	85 (9)	87 (13)	NS
κ-Casein variant	(AA, AA, AB, BB)	(AA, AA, AA, AA)	
Parity	1.75 (0.95)	1.50 (0.58)	NS
Lactation week	41.5 (32.3)	30.8 (9.19)	NS
Lactation day	290 (225)	213 (64)	NS
Time since last milking (h)	9.66 (3.15)	11.24 (0.58)	NS
Milk yield (kg)	11.90 (3.78)	15.87 (9.19)	NS
Daily milk yield (kg)	30.42 (10.35)	29.08 (64.33)	NS
pH	6.71 (0.05)	6.74 (2.62)	NS
Conductivity (mS/cm)	4.54 (0.14)	4.88 (3.72)	NS
Casein micelle size ² (nm)	199.4 (10.7)	222.7 (10.9)	NS
Fat (g/100 g)	4.12 (0.79)	3.41 (0.09)	NS
Protein (g/100 g)	3.47 (0.29)	3.23 (0.29)	†
Casein (g/100 g)	2.69 (0.24)	2.48 (22.78)	NS
Urea (mM)	3.97 (0.97)	3.81 (0.32)	*
Casein number (casein%/protein%)	0.78 (0.01)	0.77 (0.19)	†
Citric acid	0.18 (0.03)	0.19 (0.17)	†
Lactose (g/100 g)	4.90 (0.05)	4.80 (0.76)	NS
SCC (× 1,000/mL)	398.75 (596)	37.75 (0.01)	NS
Rennet coagulation time (min)	13.11 (1.65)	17.06 (0.03)	NS
Curd firming rate (Pa/min)	27.59 (2.0)	14.13 (0.07)	***
Gel firmness (G' max; Pa)	424.38 (168.48)	234.38 (25.9)	NS

¹Good = well-coagulating milk; Poor = means of values for 2 poorly coagulating samples and 2 noncoagulating samples as outlined in Figure 1.

²Hydrodynamic diameter.

† $P \leq 0.1$; * $P < 0.05$; *** $P < 0.001$.

by their mean values ($P < 0.05$) in the 3 coagulation property traits (Table 1). The PC milk samples had a noticeably weaker curd when visually evaluated at the end of analysis. Two of the cows in the PC milk group produced milk that, even after 40 min incubation with rennet, could be poured from the ReoRox cup with a fluid appearance like that of nontreated milk. These samples were referred to as NC milk samples (Figure 1) in the following detailed analyses.

The 2 cows that consistently produced NC milk were subjected to further study of milk coagulation properties at the udder quarter level to ascertain whether the coagulation properties of the composite milk differed from those of milk at the quarter level. The coagulation properties of the quarter milk samples were not significantly different from those of the composite milk sample (result not shown).

Milk Composition

The analyzed milk composition and production traits of cows producing WC (n = 4) and PC (n = 4) milk

are given in Table 1. The low number of samples in the groups and the fact that 2 of the cows in the PC group in Table 1 were actually producing NC milk resulted in large standard deviations for some of the parameters listed in Table 1. The 2 groups of cows differed significantly or tended to differ ($P < 0.1$) in the contents of total protein, urea, and citrate, with total protein being higher in the WC group, whereas urea and citrate were higher in the PC group. In addition, the levels of total, micelle-bound, and soluble Ca, as well as total and micelle-bound P, were higher in the WC group. To study whether the ratio of micelle-bound Ca and P to total CN or the ratio of micelle-bound Ca to micelle-bound P differed between coagulation groups, these ratios were calculated, but no significant differences between coagulation groups were found (results not shown). The effect of addition of Ca to the NC milk at a level comparable to that used in the dairy industry (0.005%) was furthermore studied by addition of CaCl_2 to the NC milk before the rheological analyses. Addition of Ca was found to cause a significant decrease in coagulation time, but a lesser and nonsignificant effect

was found in both gel firmness and curd-firming time, and therefore the NC milk was not improved to the same level as that of WC milk (results not shown).

Effect of Blending NC and WC Milk

The effect of blending milk from individual cows representing NC and WC milk on the resulting coagulation properties is shown in Figure 2A. The NC milk reduced the coagulation properties of the WC milk in a manner analogous to titration. With an equal percentage of the 2 milk samples in the blend, the coagulation property of the WC milk was markedly compromised, and quality was markedly deteriorated with 25% NC milk in the blend.

Results of blending a pool of WC milk with a pool of PC milk (consisting of an equal mixture of the 2 PC milk samples and the 2 NC milk samples) on the resulting coagulating properties are shown in Figure 2B. The effect of having increasing proportions of poorly coagulating milk in the blend is equivalent to the result shown in Figure 2A; the overall quality deteriorates with increasing contents of PC milk. However, an increase from 75 to 95% of PC milk in a milk pool seemed not to impose further negative changes, indicating that at least 25% WC milk must be present in a milk pool to counteract the poor coagulation properties, which was not the case for the individual milk samples.

Casein Micelle Size

The average hydrodynamic diameter of CN micelles was determined in fresh, skim milk obtained from the 8 selected cows by dynamic light scattering, and the results are given in Table 1. The samples were diluted in MUF to avoid multiple scattering while preserving the environmental conditions of the CN micelles. Milk with good coagulation properties contained CN micelles with a relatively smaller mean diameter compared with milk from cows in the PC group, but this was not statistically significant (Table 1, $P < 0.11$), which may be due to the small number of samples available for comparison. The average hydrodynamic diameter is shown in Figure 3 as a function of the relative content of κ -CN in relation to total CN as determined by liquid chromatography (LC)/ESI-MS analysis. A negative correlation ($R^2 = 0.77$) existed between micelle hydrodynamic diameter and the relative content of κ -CN in relation to total CN. Moreover, NC and PC milks had a lower content of κ -CN relative to total CN, and casein micelles in the NC and PC milks had a larger diameter.

CMP Analyses

To determine whether failure in the enzymatic step of the coagulation process causes the noncoagulation

Figure 2. Effect of blending milk with different coagulation properties. (A) Effect on curd firming rate of mixing 1 noncoagulating skim milk sample with 1 well-coagulating sample. The samples were obtained from 2 individual Holstein cows, and the well-coagulating milk was mixed with noncoagulating milk in ratios from 0 to 100% (vol/vol). (B) Effect on curd firming rate of mixing 2 milk pools, representing a pool of milk from the 4 well-coagulating milk samples and a milk pool of poorly coagulating milk (the 2 poorly coagulating samples and the 2 noncoagulating samples), as shown in Figure 1 and Table 1. The well-coagulating milk pool was mixed with the poorly coagulating milk pool in ratios from 5 to 95% (vol/vol). The measurements were carried out in duplicate. RCT = rennet coagulation time.

phenomenon observed for the 2 NC milk samples, we evaluated the generation of CMP by RP-HPLC analyses. Caseinomacropeptide was isolated from supernatants of acid-precipitated milk samples at different time points after addition of chymosin to WC or NC milks.

Figure 3. Average hydrodynamic diameter (nm) of casein micelles from well-coagulating ($n = 4$), poorly coagulating ($n = 2$), and noncoagulating ($n = 2$) milk in relation to the relative content of κ -CN to total CN. The average hydrodynamic diameter of the casein micelles was determined by dynamic light scattering as 5 replicate measurements of fresh skim milk samples diluted in milk ultrafiltrate. Note the offset of the axes.

As evident from Figure 4, the addition of chymosin caused rapid generation of CMP, which was within the same range in all samples in all coagulation property groups, as shown at 0 and 25 s in Figure 4. This shows that CMP was released and κ -CN cleaved by chymosin in all samples, including the 2 NC samples.

Milk Proteomic Profiling by LC/ESI-MS

As protein composition and milk protein genotypes are important properties for coagulation properties, the protein profiles of the skim milk samples were studied using a reversed phase liquid chromatography-based method, where the genotypes and posttranslational modifications of selected proteins were identified with the use of ESI/MS.

A chromatogram of bulk milk is shown in Figure 5A. The milk proteins eluted in the order κ -CN, α_{S2} -CN, α_{S1} -CN, β -CN, β -LG, and α -LA. Protein elution time was found to be dependent on both genotype and degree of posttranslational modification. κ -Casein eluted in 3 major peaks (Figure 5A, peaks a to c). Peak (a) contained different glycosylated forms of κ -CN [mean molecular weight (M_w) $>19,000$ Da]; peak (b) contained nonglycosylated κ -CN-A (mean $M_w = 19,035$ Da); and (c) contained nonglycosylated forms of κ -CN-B (mean $M_w = 19,003$ Da). The α_{S2} -CN eluted in 2 major peaks (Figure 5A, peaks d and e) and several minor peaks corresponding to varying states of phosphorylation. The degree of phosphorylation varied from 11P to 13P, and the elution time was found to increase with an increas-

ing level of phosphorylation. Peak (d) corresponded to α_{S2} -CN-11P (mean $M_w = 25,226$ Da) and (e) to α_{S2} -CN 12P (mean $M_w = 25,306$ Da). A minor shoulder on the right side of peak (e) was identified with an additional phosphate group; that is, α_{S2} -CN-13P (mean $M_w = 25,386$ Da). The α_{S1} -CN separated into a major and a minor component (Figure 5A, peaks f and g). Peak (f) contained the major component, α_{S1} -CN-8P (mean $M_w = 23,613$ Da), whereas peak (g) contained the minor component, α_{S1} -CN-9P (mean $M_w = 23,692$ Da).

Three different genotypes of β -CN were identified (Figure 5A): peak (h) represented β -CN B (mean $M_w = 24,087$ Da), peak (i) represented β -CN A¹ (mean $M_w = 24,019$ Da), and peak (j) represented β -CN A² (mean $M_w = 23,978$ Da). The major whey proteins α -LA and β -LG, which are not phosphorylated, eluted after the caseins. In bulk milk, 2 β -LG genotypes were identified in Figure 5A, peaks (k) and (m), corresponding to β -LG B (mean $M_w = 18,280$ Da) and β -LG A (mean $M_w = 18,366$ Da), respectively. The mobility of α -LA (mean $M_w = 14,184$ Da) was highly dependent on temperature, but at the temperature of 35°C used here, α -LA eluted between β -LG-A and β -LG-B (Figure 5A, peak l).

Protein Profiling of Individual Cow's Milk

Milk from 8 cows was investigated with respect to genotype and posttranslational modifications of the caseins. Chromatograms of the 8 individual milk samples

Figure 4. Generation of caseinomacropeptide (CMP) in well-coagulating, poorly coagulating, and noncoagulating milk samples before and after chymosin addition. The CMP content was determined in milk samples before addition of chymosin (wo chymosin) and 25 s after chymosin addition (chymosin). The CMP content represents the ratio of integrated CMP peaks in the sample relative to that of the standard (Lacprodan CGMP-20, Arla Foods amba, Global Ingredients, Nr. Vium, Denmark) as determined by reverse phase-HPLC.

Figure 5. Separation of milk proteins by reverse phase-HPLC. Chromatograms of (A) bulk milk and (B) individual milk with good, poor, and noncoagulation. Protein identification was achieved by molecular mass determination by use of mass spectrometry. (a) Glycosylated forms of κ -CN A and B; (b) nonglycosylated κ -CN-A; (c) nonglycosylated κ -CN-B; (d and e) α_{S2} -CN in different states of phosphorylation; (f and g) α_{S1} -CN in different states of phosphorylation; (h, i, and j) different genetic variants of β -CN; (k and m) β -LG A and B, respectively; and (l) α -LA. Abs = absorbance.

are shown in Figure 5B. The κ -CN, β -CN, and β -LG genotypes of the 8 cows are shown in Table 2. Of the 8 cows, 6 had the AA genotype for κ -CN, whereas the other 2 had the AB and BB genotypes. For β -CN, 4 cows had the A¹A² genotype, whereas 3 cows had the A²A² genotype, and 1 cow had the rare A¹H genotype, according to the mass determination (Senocq et al., 2002). All 8 cows had the AB genotype for β -LG.

In addition to the identification of genotypes, the chromatograms revealed interesting information about posttranslational modifications of the caseins in relation to coagulation properties. In Figure 6, represen-

tative chromatograms of 2 milk samples (representing WC and NC properties) have been enlarged in the elution interval of α_{S2} -CN (Figure 6A) and α_{S1} -CN (Figure 6B). Although they are not completely separated, it is possible to distinguish between different phosphorylation forms of α_{S2} -CN and α_{S1} -CN; α_{S2} -CN-11P was more abundant compared with α_{S2} -CN-12P in WC milk, whereas the situation was reversed for NC milk, in which the 12P form was elevated. The same trend was seen for α_{S1} -CN, where α_{S1} -CN-9P accounted for a lower proportion of the total α_{S1} -CN in the WC milk compared with the NC milk, with a simultaneous, relative increase in α_{S1} -CN-9P of the NC milk.

To compare the level of phosphorylation of the α -caseins in WC, PC, and NC milks, the ratio of peak area of α_{S2} -CN-11P to the total peak area of α_{S2} -CN was used as a measure for the fraction of the low phosphorylated form to the total content of α_{S2} -CN. Similarly, for α_{S1} -CN, the ratio of the peak area α_{S1} -CN-8P to the total area of α_{S1} -CN was calculated. These fractions of low phosphorylated forms to total α_{S1} -CN or α_{S2} -CN in the 8 individual milk samples are presented in Table 2. Interestingly, WC milk appeared to contain a larger proportion of variants with a lesser degree of phosphorylation compared with PC and NC milk. Similarly, the degree of glycosylation was investigated by comparing the ratios of the area of the glycosylated κ -CN peak with the total area of κ -CN (data not shown); however, no apparent differences were observed between milk samples.

DISCUSSION

This investigation showed the effect of variations in protein composition, level of phosphorylation of α_{S1} - and α_{S2} -CN, ionic content, fraction of κ -CN relative to total CN content, and casein micelle size on the coagulation properties of milk from individual cows. Studies of variation in the composition of individual milk samples in relation to coagulation properties are numerous, whereas studies on the composition of NC milk and on the effect of mixing milk with divergent clotting properties are few. We observed in this study that both RCT and curd firming rate were impaired in WC milk by addition of increasing amounts of NC milk (Figure 2A); a significant effect on both properties was observed at 25% NC milk occurrence. Vice versa, by increasing the proportion of WC milk relative to NC milk, the coagulation properties of NC milk are somewhat restored, albeit not to the level of WC milk. Looking at the effect of blending milk pools of WC milk with a pool of PC milk (a mixture of PC and NC milks; Figure 2B), we observed that a 50% mixture was not sufficient to restore completely the coagulation

Table 2. Genotypes of milk proteins and degree of phosphorylation of caseins

Coagulation class ¹	Milk protein ²			Fraction of low phosphorylated forms ³ in:	
	κ -CN	β -CN	β -LG	α_{S2}	α_{S1} ⁴
Good	AA	A ¹ A ²	AB	0.43	0.76
Good	BB	A ¹ H ⁵	AB	0.46	0.75
Good	AA	A ¹ A ²	AB	0.48	0.75
Good	AB	A ¹ A ²	AB	0.42	0.73
Poor (non)	AA	A ² A ²	AB	0.32	0.66
Poor (non)	AA	A ² A ²	AB	0.37	0.72
Poor	AA	A ² A ²	AB	0.31	0.67
Poor	AA	A ¹ A ²	AB	0.28	0.73

¹Good = well-coagulating milk; Poor (non) = 2 poorly coagulating samples and 2 noncoagulating samples as outlined in Figure 1; Poor = poorly coagulating milk.

²Nomenclature as described in Farrell et al. (2004); genotypes as determined from the molecular mass of the protein.

³Fraction of low-phosphorylated form of α_{S2} -CN was determined as the ratio of the area of α_{S2} -CN-11P to the total area of α_{S2} -CN; fraction of low-phosphorylated form of α_{S1} -CN was determined as the ratio of the area of α_{S1} -CN-8P to the total area of α_{S1} -CN.

⁴Based on a measured mass of 23,963 Da, the genotype was assigned as variant H, according to Senocq et al. (2002).

properties of PC milk, which is in agreement with the observations of Okigbo et al. (1985b).

In agreement with the Okigbo et al. (1985b) and Hallén et al. (2010), the present study found, however, that the milk clotting properties of PC milk could be improved by addition of calcium chloride, albeit not to the level of WC milk. Hallén et al. (2010) observed that to improve the coagulation properties of NC milk to the level of that in WC milk, Ca should be added to a level of 0.05%. As more than 0.02% added CaCl₂ is not recommended in commercial cheese making, this is an important reason to survey and prevent too high frequencies of PC milk, and to prevent dairy cows that consistently produce NC milk from supplying milk to the dairy industry for cheese production. These findings give strong reasons for including coagulation properties as a parameter in future breeding programs of dairy cows.

The 2-step nature of the coagulation process led us investigate whether the first enzymatic step failed in the milks with impaired rennet ability. As evident from Figure 4, CMP was equally released in all milks after rennet addition, and hence the impaired coagulation ability was not caused by a nonfunctional error in the enzymatic cleavage step, in agreement with earlier reports (van Hooijdonk et al., 1986). We further observed that PC and NC milks were characterized by a low total content of κ -CN relative to total CN and by larger casein micelles, and concluded, in accordance with the study of Hallén et al. (2010), that a low content of κ -CN is a risk factor for noncoagulation, because it is negatively correlated with casein micelle size. The connection between micelle size and coagulation properties

was found to be in agreement with the literature; that is, milk with small casein micelles aggregates faster and forms a firmer curd than milk with large casein micelles (Glantz et al., 2010).

It has been observed that milk with impaired clotting properties, and NC milk, could be sampled from cows in early lactation, and the impaired clotting properties could be due to differences in composition and distribution of milk salts (Okigbo et al., 1985b,c). The NC phenomenon has also been observed in healthy cows in mid lactation (Ikonen et al., 2004). In the present study, we were not able to show a significant difference in ionic distribution between milks with good and poor coagulation properties. This is assumed to be due to the small amount of material available, but differences in the ionic content between coagulation groups were observed, with higher mean levels of micelle-bound Ca and P in the WC milk samples. Cows with mastitis are known to be associated with PC or NC milks (Okigbo et al., 1985a). However, we found no difference in coagulation properties of quarter milk samples compared with the composite milk sample, indicating that the noncoagulation and poor coagulation traits of composite milk were not caused by the quality of milk from a single quarter. As mastitis or elevated cell count is often associated with a single udder quarter, this shows that the NC milks in this study were not triggered by milk quality in a single udder quarter, as could, for example, be caused by subclinical mastitis.

The different genetic milk protein variants and casein haplotypes have a major effect on the protein composition of milk. In general, the A allele of κ -CN has been associated with a longer RCT and a weaker curd

Figure 6. Posttranslational modifications of α_{S2} -CN and α_{S1} -CN in well-coagulating and noncoagulating milk. The 3 major α_{S2} -CN peaks correspond to modification of the protein with 11, 12, or 13 phosphate groups. α_{S1} -Casein eluted in 2 major peaks corresponding to modifications with 8 or 9 phosphate groups. Abs = absorbance.

(Hallén et al., 2007). In the present study, all cows in the PC group had the AA κ -CN genotype (Table 2). Interestingly, of the 4 cows that produced NC or PC milk, 3 of them were genetically related to each other; that is, they had the same sire. This finding supports the hypothesis that this phenomenon is caused, to some extent, by a genetic factor, as previously reported (Tyrisseva et al., 2004). However, in the group of cows producing milk with good coagulation properties, 2 further κ -CN AA variants were found. This shows that κ -CN AA is a contributing risk factor for the occurrence of PC and NC milk, but is not sufficient in itself. Tight genetic linkage exists between the major milk

proteins; therefore, the effect of the composite genotypes, or haplotypes, is more relevant to consider than the single-locus genotypes (Mayer et al., 1997; Comin et al., 2008; Heck et al., 2009). Regarding the genetic variants of β -CN and κ -CN, 3 of the 4 cows in the PC groups had the unfavorable haplotype A²A²/AA, which is associated with a lower content of κ -CN (Hallén et al., 2008). This haplotype, combined with the presence of the more phosphorylated variants of α_{S1} - and α_{S2} -CN, may result in an increased risk for NC milk occurrence. We have shown here that a higher proportion of the α_{S1} -CN-9P to α_{S1} -CN-8P, and furthermore of α_{S2} -CN-12P and 13P relative to 11P in the NC milk. In agreement with this observation, casein variants with lower charge have been reported to improve coagulation through facilitated aggregation (Feagan et al., 1972). This is in agreement with the pair potential model for the second phase of coagulation proposed by Tuinier and de Kruif (2002), in which aggregation is influenced by steric repulsion of brush border κ -casein, electrostatic repulsion (phosphorylated casein), and van der Waals attractions. Interestingly, mapping of chromosomal regions underlying NC milk in Finnish Ayrshire has indicated 2 potential candidate genes, a serine/threonine kinase on chromosome 2 and a sialyl transferase, which catalyzes the last step of glycosylation of κ -CN, on chromosome 18 (Tyrisseva et al., 2008). This finding supports the observation in the present study that posttranslational modifications of the caseins may be one of the underlying causes for NC milk. It has been reported that the nonglycosylated variant of κ -CN is only partly cleaved by chymosin (Lieske and Valbuena, 2008), but this requires further studies and was not part of the present investigation.

CONCLUSIONS

In a screening study of 53 Danish Holstein-Friesian dairy cows, 4% were observed to consistently produce NC milk. The NC milk was not the result of very poor milk quality of a single udder quarter. Mixing NC cow milk samples with WC milk samples decreased the coagulation of the WC milk in a manner analogous to titration, with clear deterioration of coagulation properties at the level of 25% NC milk. Failure in the first step of milk coagulation (i.e., cleavage of κ -CN) was not the underlying reason for the NC milk phenotypes, because CMP was released after addition of chymosin. Instead, the risk factors for NC centered on the second phase of milk coagulation. The NC milks were characterized by larger casein micelles and a low content of κ -CN relative to total CN. The κ -CN AA variant, associated with a lower content of κ -CN, is a risk factor for NC milk, but is not sufficient in itself.

This genotype, combined with the presence of the more phosphorylated variants of α_{S1} - and α_{S2} -CN, may result in an increased risk for occurrence of NC milk, which contained greater fractions of α_{S1} -CN-9P to α_{S1} -CN-8P and of α_{S2} -CN-12P and 13P relative to α_{S2} -CN-11P.

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